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14. ABSTRACT

We are creating an anti-microbial "sense-and-destroy" system by engineering sentinel/killer cells that detect the presence of pathogenic bacteria, report the identity of the pathogen with a coded output signal, and secrete specific bacteriocins/lysins that destroy the pathogen. Engineering cells for detection and targeted destruction of pathogens offers a single integrated solution to eradicate multiple threats with an approach that is a rapid, selective, and highly sensitive. We are engineering a single cell type that detects chemical signals naturally produced by different classes of pathogens and initiates multiple specific responses, each tailored to particular members of the classes. The system will thus be able to analyze environmental conditions and execute an "intelligent" response by utilizing multiple, customized treatments.

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Scientific and Technical Objectives

We are creating an anti-microbial "sense-and-destroy" system by engineering *sentinel/killer* cells that detect the presence of pathogenic bacteria, report the identity of the pathogen with a coded output signal, and secrete specific bacteriocins/lysins that destroy the pathogen. Engineering cells for detection and targeted destruction of pathogens offers a single integrated solution to eradicate multiple threats with an approach that is a rapid, selective, and highly sensitive. We are engineering a single cell type that detects chemical signals naturally produced by different classes of pathogens and initiates multiple specific responses, each tailored to particular members of the classes. The system will thus be able to analyze environmental conditions and execute an "intelligent" response by utilizing multiple, customized treatments. Additional advantages of the proposed approach include the following:

• The sentinel/killer cells will function without human intervention, and may therefore be deployed in remote or access-compromised environments including, for example, water supply systems. They may be embedded and remain inert until threat is detected, and will then serve as an early detection and rapid response system.

• Pathogen detection will be highly sensitive due to the discriminating nature of cells and our ability to construct signal amplifying genetic circuits.

• This strategy will be effective against antibiotic-resistant pathogen strains.

• Engineered sentinel cells may serve as a high-throughput screening platform for the discovery of the next generation of antibiotics.

Approach

Selective and sensitive pathogen detection is being achieved through engineering of sentinel circuits in recombinant *E. coli* cells with components of canonical quorum sensing (QS) signaling pathways. These pathways are normally used by bacteria to sense, in a species-specific manner, their population density and induce population-wide responses at specific density levels. QS Signaling pathways also control the secretion of virulence factors by pathogens.

We are engineering prototype sentinel circuits for targeted detection of gram-negative and gram-positive pathogens. The prototypes use elements from the Lux/IR QS system of *Pseudomonas aeruginosa* for gram-negative detection and the peptide-mediated Com QS system of *Bacillus subtilis* for gram-positive detection. Together these two prototype sentinel circuits cover a wide range of important intracellular pathogens with similar QS systems. The sentinel circuits that are under developement for detecting *P. aeruginosa* and *B. subtilis* will be interfaced with circuits that employ highly selective lysins to destroy the pathogens.

Concise Progress Summary

We are able to engineer Sentinel cells both for gram-negative and gram-positive pathogens. We have engineered *E. coli* cells that can respond effectively to the presence of gram-negative pathogen, *Pseudomonas Aeruginosa*. We are able to engineer them in such a way that they sacrifice themselves in order to release the killer proteins, which then kills the pathogen. This signifies that the sentinels are ready to be armed. Simultaneously we have been able to successfully demonstrate a difference in the killing activity of an exogenously added antimicrobial peptide, CDAP-4, towards *P. aeruginosa* and *E. coli*. We are currently in the process of constructing receivers for a gram-positive pathogen, *Bacillus subtilis*.

Expanded Accomplishments

Gram-negative Pathogen Detection

a) Accomplishments

In the canonical gram-negative Quorum Sensing system, an I-protein synthase produces acylated homserine lactone (AHL) autoinducers which diffuse freely between the cytoplasm and the environment, then directly interact with R-protein transcriptional regulators to control the expression of target genes. The design for sentinel/killer cells involves an R-protein (regulated by an inducible promoter) which binds to 3OC12HSL and promotes expression of E/GFP and lysin. We grew *P. aeruginosa* to different OD's and aliquot the supernatant. The concentration of 3OC12HSL in the supernatant is directly proportional to the pathogen density. The filter sterilized supernatant contains all the signals the pathogen uses for quorum sensing. We then grew our 3OC12HSL-responsive receiver cells in the supernatant and measured their fluorescence. The graph in Fig.1 demonstrates the change in fluorescence based on pathogen density, indicating that response of the sentinel cells depends on the density of the pathogen.

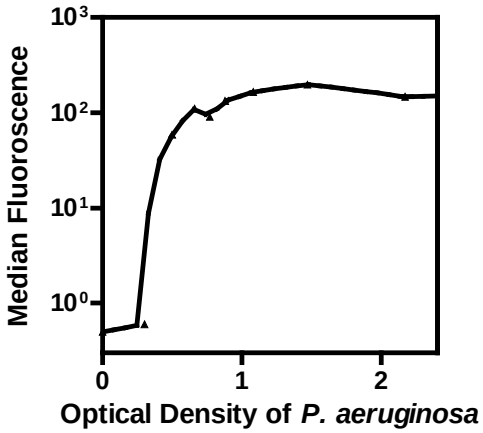

Figure 1. Fluorescence of receivers as a response to the pathogen density

Bacteriocins are highly specific and potent toxins produced during stressful conditions and result in rapid elimination of neighboring cells which are not immune to their effect. A small portion (depending on the invasion dynamics) of the cells produces bacteriocins under stressful conditions, such as nutrient depletion, overcrowding, stationary phase of growth, high temperatures etc. They have evolved to parasite various cell surface receptors that are normally involved in the uptake and passage of small nutrient molecules such as Vitamin B12 across the OM. BtuB is the receptor protein in E.coli. We have engineered bacteriocins to specifically kill pathogen, PAO1. Colicins (E1) and Pyocins (S2) are naturally expressed lysins in

gram-negative bacteria. E1 and S2 allow these bacteria to gain an advantage over other species by specifically destroying other bacterial strains. Colicins usually have three distinct domains, Fig. 2, with different functionality.

Figure 2. Various domains of a typical Colicin

The Recognition Domain recognizes the specific receptors on the surface of the target species. The Translocase Domain translocates the Nuclease domain into the cell. This Nuclease Domain can be DNase or RNase and it kills the cell by cleaving the DNA or RNA of the cell. The producing cell is protected from its own colicin by two mechanisms. Firstly the Recognition Domain can form complex only with the receptors of the sensitive cell and hence it does not bind to the producing cell surface receptors. Secondly colicins are produced along with an immunity protein which is translationally coupled to the colicin and forms a tight complex with the nuclease domain. This complex disassociates only when the Receptor domain binds to the corresponding receptor on the cell surface. This dual protection provides the producing cell evolutionary advantage over the sensitive cells, which lack the immunity protein. Fig. 3 explains the schematic representation of the colicin killing

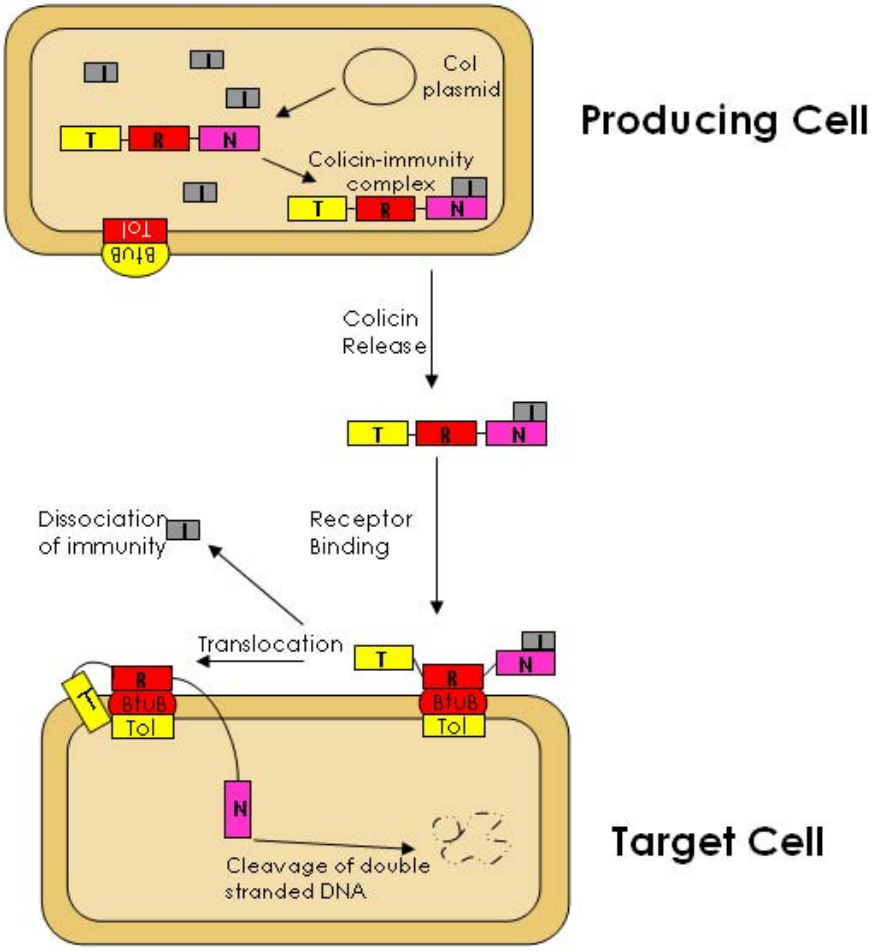

Figure 3. Schematic representation of killing by Colicin

Like Colicins the bacteriocins in P.aeruginosa are Pyocins. They have the same structure as Colicins. To get a protein which selectively kills PAO1, we replaced the colicin Recognition and Translocation domains to that of Pyocin, as illustrated in Fig. 4.

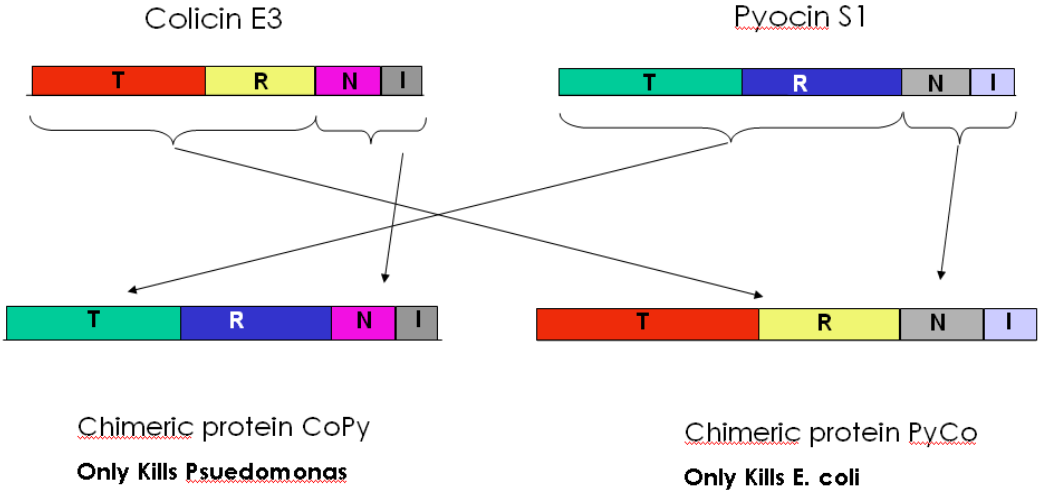

Figure 4. Engineering of Chimeric Proteins

The modified protein called CoPy only recognizes the cell surface receptors of P. aeruginosa and translocates the nuclease domain into the pathogen, leaving the sentinel cells intact. Fig. 5 describes the functioning of CoPy. It is interesting to note that the modified protein only kills the pathogen while the sentinel is unaffected by it because of the immunity protein and different cell surface receptors.

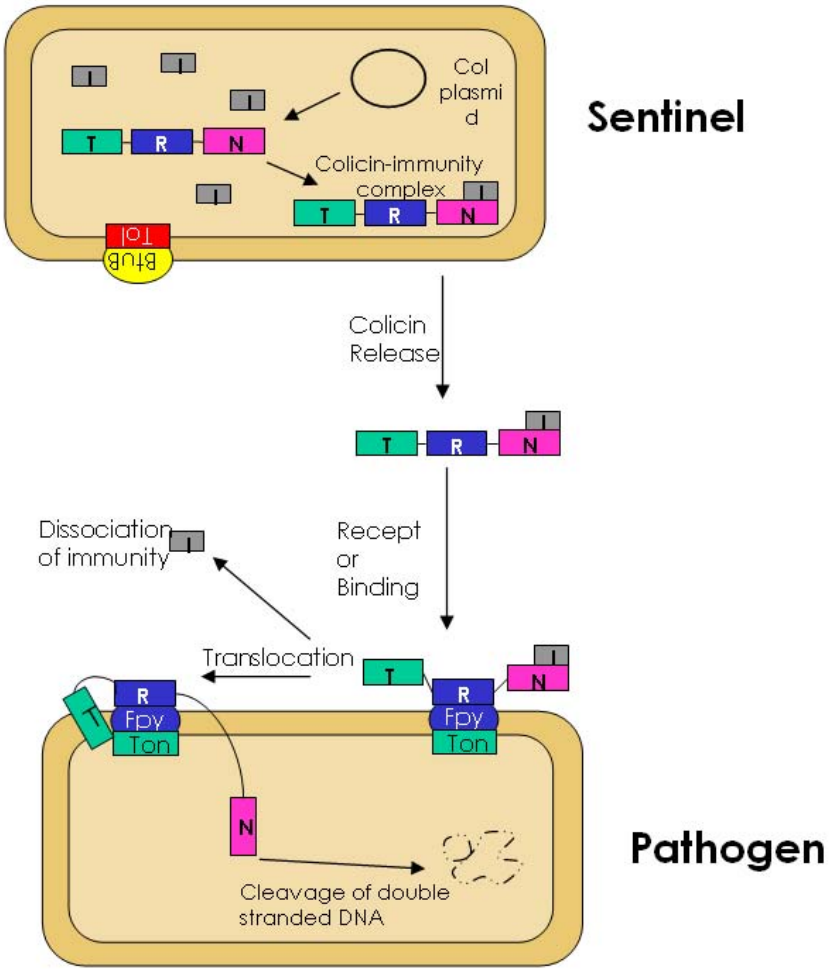

Figure 5. Schematic representation of killing by CoPy

We tested the effects of CoPy on the sentinel cells when produced. CoPy was cloned under the control of PltetO1. We grew the sentinel cells (100ng/ml aTc induced) producing CoPy and cells without CoPy (containing pProtet plasmid) and monitored their OD for about 20 hours. The graph in Fig. 6 shows that the CoPy inside does not kill the sentinels.

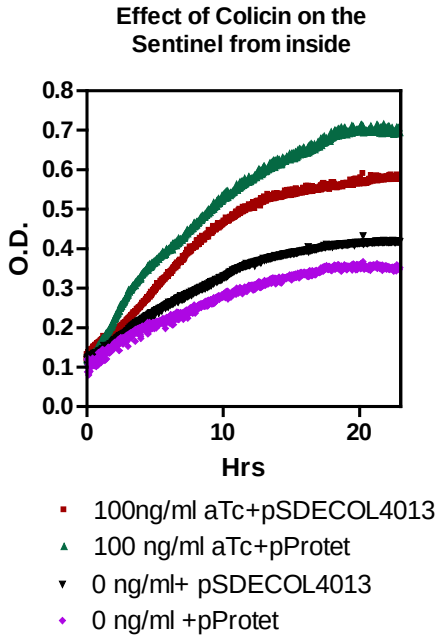

Figure 6. Graph illustrating that the sentinels are unaffected by the CoPy when produced

We purified CoPy using HisTag fused at the N-terminal of the protein. To see the effect of CoPy, we incubated the sentinel cells (170uL of OD 0.23) and PAO1 (170uL of OD 0.23) with 30uL of purified CoPy for about 20 hours. As a control the cells were also incubated with equal amount of PBS and Background Buffer used for purifying CoPy.

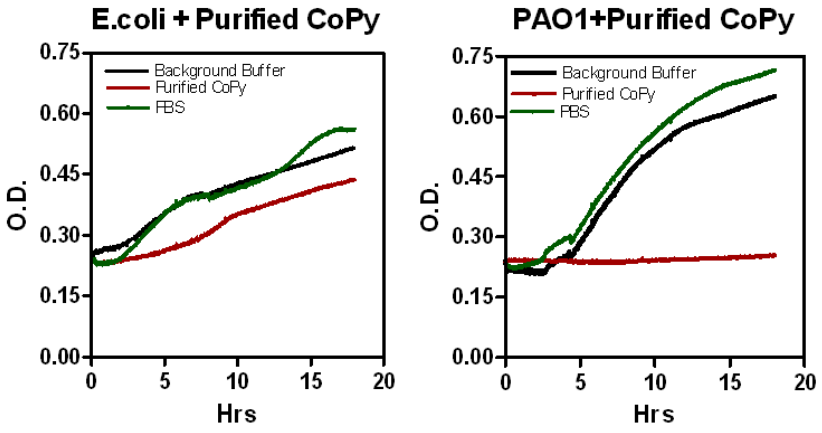

Figure 7. Graph illustrating that the CoPy only affects the pathogen leaving the Sentinels intact

Fig. 7 shows that CoPy has stopped the PAO1 from growing completely, while the sentinel's growth is not affected. The Sentinels and PAO1 were incubated in the cell lysate containing CoPy(Sentinel Lysate) and pProtet Lysate as a control.

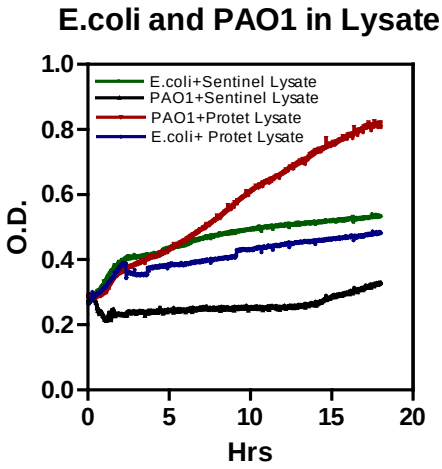

Figure 8. Effect of sentinel cell lysate on the Sentinels and the Pathogen

Fig.8 again emphasizes that the sentinels grow and PAO1 dies in the lysate containing CoPy. To release the lysin (killer protein) from *E. coli* killer cells, we need to coordinate pathogen sensing with a secretion mechanism that can deliver lysin to the medium which in turn kills the pathogen. One way to achieve this is by co-expressing the lysis protein E (obtained from *E. coli* bacteriophage phiX174). We built sentinel cells which respond to the presence of 3OC12HSL by expressing E-protein.

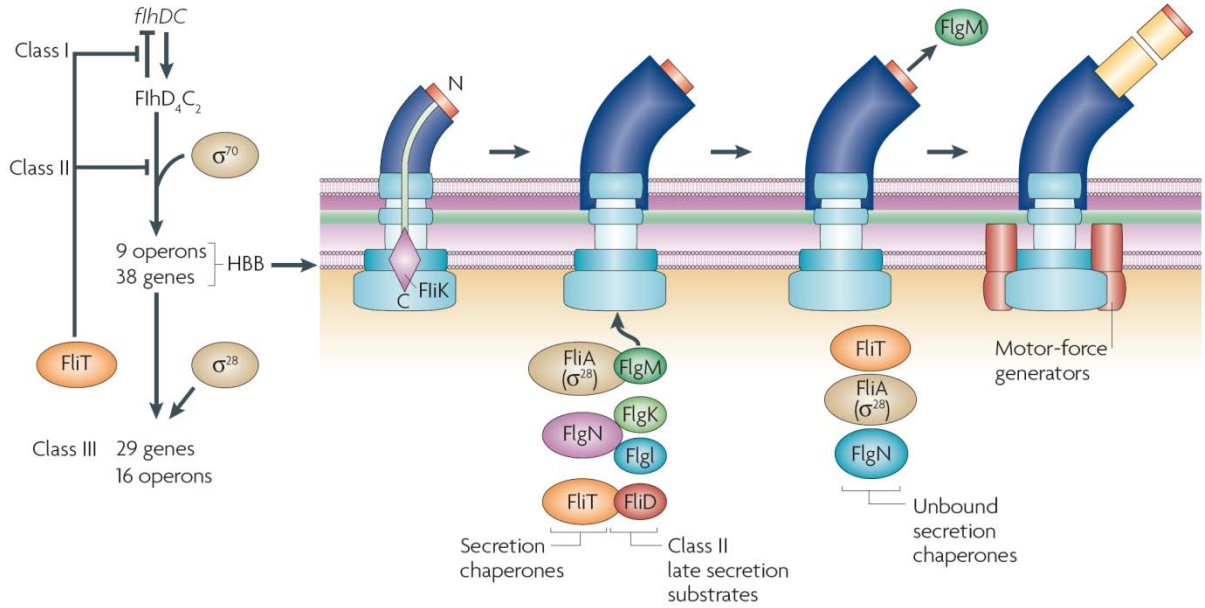

Figure 9. Coupling of flagellar gene regulation to flagellum assembly. Figure adapted from [Chevance and Hughes, Nature Reviews, 2008]

In *S. typhimurium*, (as explained in the above Fig. 9) the flagellar master operon *flhDC* is at the top of this hierarchy and controls the fundamental decision of whether to produce flagella. The *flhDC* operon is expressed from a class I promoter. The FlhD and FlhC proteins form a heteromultimeric complex (FlhD4C2) that functions as a transcriptional activator to promote σ70-dependent transcription from the class II flagellar promoters. The class II promoters direct transcription of the genes that are needed for the structure and assembly of the flagellar motor structure, which is also known as the HBB. Upon HBB completion, class III promoters are transcribed by σ28 RNA polymerase, which is specific for flagellar class III promoters. Prior to HBB completion, σ28 RNA polymerase is inhibited by the anti-σ28 factor FlgM. Upon HBB completion, FlgM is secreted from the cell, presumably through the completed HBB structure, and σ28-dependent transcription ensues. In this way, genes such as the flagellin filament genes, the products of which are needed after HBB formation, are only transcribed when there is a functional motor onto which they can be assembled. E.coli has essentially similar flagellar control. We fused CoPy to FlgM. The fused protein is under the transcriptional control of Lux promoter. This promoter is transcribed only when there is pathogen in the medium. Figure 9 shows the western blot of CoPy fused to FlgM. Clearly we are able to secrete CoPy with the help of FlgM. The next step is to verify the specific activity of the fused protein.

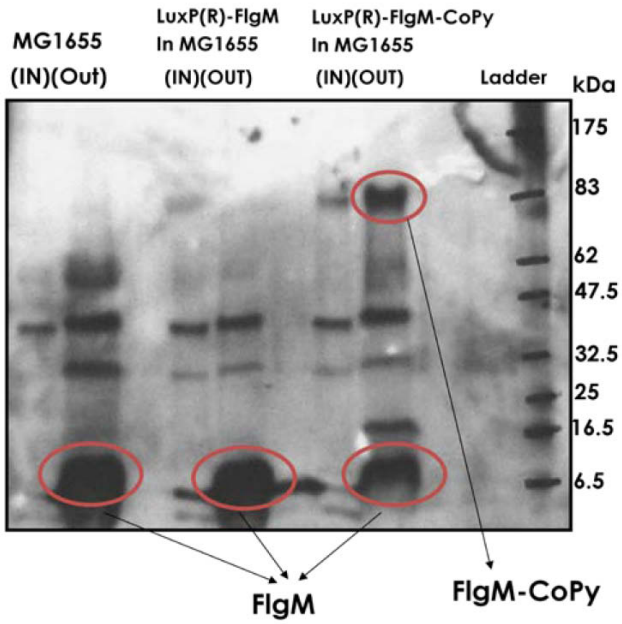

Figure 9: Western Bolt showing the secretion of FlgM-CoPy fusion.

Alternatively we are incorporating a novel peptide secretion mechanism that hijacks the flagellar type III secretion apparatus. The product of gene *fliC* acts as a cap on the flagellar hook on which the whole flagella is built. A *fliC* deficient knockout strain will secrete peptides

that are expressed between the 5'UTR and 3'UTR sequences of *fliC*. We are currently building a strain that secretes the lysin through this mechanism to avoid cell suicide.

b) Significance

The above results demonstrate our capability to effectively sense the pathogen in the medium and kill it. We engineered bacteriocins to kill the pathogens selectively. We are working on integrating these killer proteins with an efficient technique to secrete these lysins into the medium once the pathogen is detected.

Gram-Positive Pathogen Detection

a) Accomplishments

In the canonical gram-positive QS mechanism, autoinducers are modified oligopeptides, and a two-component signal transduction system facilitates recognition and response. A membrane- bound receptor histidine kinase detects and relays the signal to a cytoplasmic response regulator, which controls target gene expression. The Com system from *B. subtilis* was chosen for the design of a prototype sentinel circuit in recombinant *E. coli*. Figure 1 shows the components of two types of gram positive QS systems that use AIP's (auto inducing peptides) as QS signals.

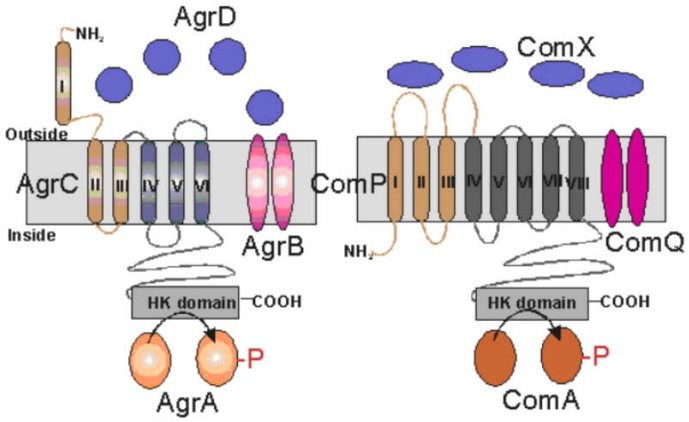

Figure 11: Gram positive QS systems. **Agr QS** of Staphylococcus aureus (left) and **Com QS** of Bacillus subtilis.

Following the successful implementation of such a sentinel circuit, it is planned to extend this circuit with *B. subtilis* specific lysins (XlyA, BlyA) to demonstrate destruction of *B. subtilis* cells in response to *B. subtilis* specific QS molecules sensed by the recombinant *E. coli* sentinel cells (Fig. 11).

We have cloned the components of both the *B. subtilis* Com and *S. aureus* Agr QS (as an alternative system) and assembled the components in a series of constructs to create *E. coli* sender and receiver cells. Although we initially chose EGFP as reporter, we are now using β-galactosidase (LacZ) as the reporter because of its simple visual and spectrophotometric detection. Fig. 12 below shows an overview of the systems engineered so far.

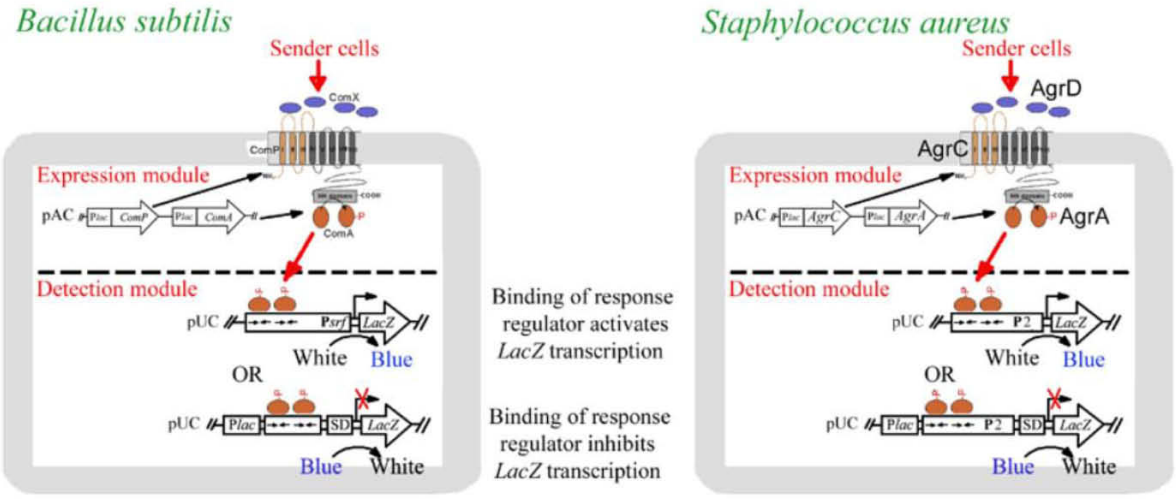

Figure 12: Overview of engineered Com and Agr QS systems.

We demonstrated that the constructed *E. coli* receiver cells secrete the B. subtilis autoinducing peptide (AIP). In addition, we have shown that the components of the *B. subtilis* Com QS system are expressed in *E. coli*. However, despite soluble expression of Com proteins and secretion of AIP by *E. coli* sender cells, no signal was generated by the receiver cells. We conducted a number of additional experiments to identify the problem. We created (via the introduction of domain truncations and in vitro evolution) a constitutively active ComA to test binding to the Psrf promoter independent of phosphorylation. One variant was obtained that surprisingly induced the Agr system but not the Com system. As outlined below in more detail, we concluded from gel shift assays that the constitutive ComA variant indeed binds to the P3 promoter normally recognized by AgrA, but does not bind to the Psrf promoter. As discussed below failure of binding the Psrf promoter is the result of missing binding elements in the promoter that were only very recently (mid July of 2008) described. Efforts are therefore underway to construct a new ComA binding consensus sequence based on these new findings.

1. Construction of Com *E. coli* sender cells

E. coli cells overexpress ComX (encoding the autoinducing peptide (AIP)) and the membrane transporter/processing protein ComQ on pUC-ComZ-ComQ secreted *B. subtilis*

AIP into the medium. The AIP was extracted from culture supernatant, purified by preparative HPLC and secretion of mature AIP in *E. coli* verified by mass spectrometry. Fig. 13 shows AIP purification by HPLC.

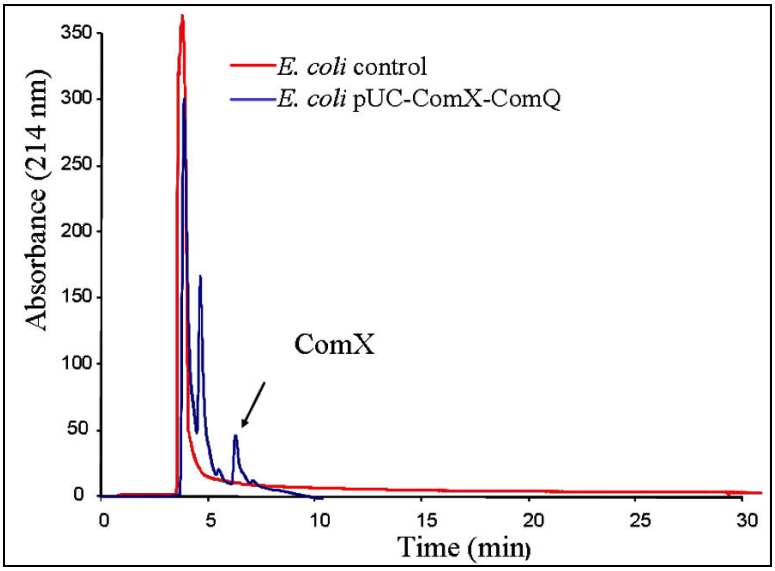

Figure 13: HPLC analysis of *B. subtilis* AIP produced by recombinant *E. coli*. ComX peak was collected and used in experiments with recombinant *E. coli* receiver cells.

2. Construction of Com *E. coli* receiver cells.

E. coli receiver cells were constructed that express on one plasmid the response regulator ComA and AIP receptor kinase ComP and on a second plasmid LacZ either under positive control of the originally described ComA regulated surfactin promoter P*srf* or under negative control of the P*lac* where the ComA binding elements of the surfactin promoter are located between P*lac* and the LacZ reporter gene. Fig. 14 shows the constructs made and which of the constructs could be induced upon induction of LacZ expression (blue cell phenotype when X-gall added to cultures or plates) by IPTG. Addition of ComX (AIP) did not result in repression (negative control constructs) or induction (positive control constructs) from P*lac*.

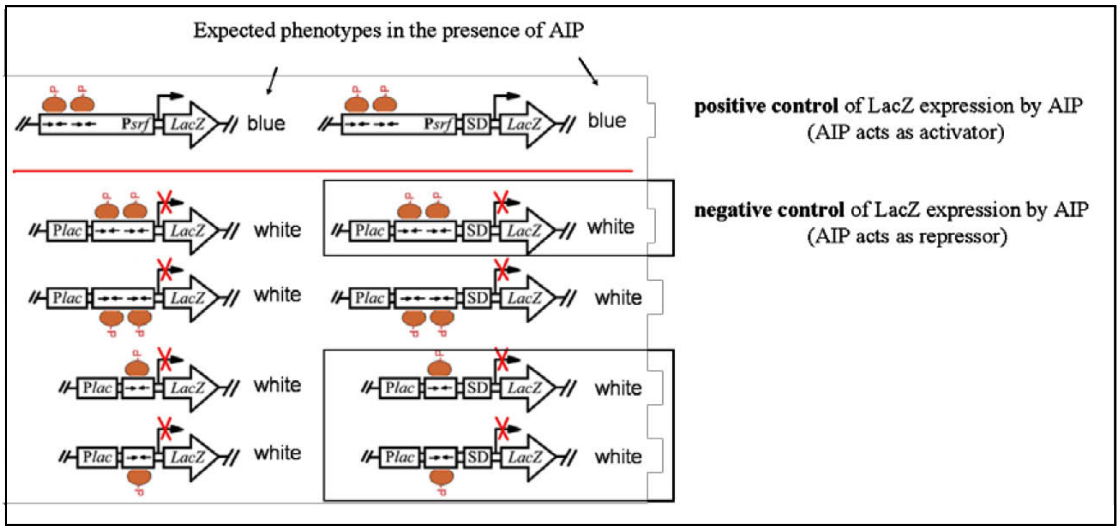

Figure 14: Promoter constructs with ComA binding boxes of the *B. subtilis* surfactin promoter.

3. Trouble shooting the recombinant Com system

We have shown that all components of the Com system are expressed in *E. coli*. The response regulator ComA is readily overexpressed as soluble protein in *E. coli*. ComP expression in *E. coli* is only tolerated at low levels (otherwise toxic) and requires the substitution of ATG to the original TTG start codon of ComP.

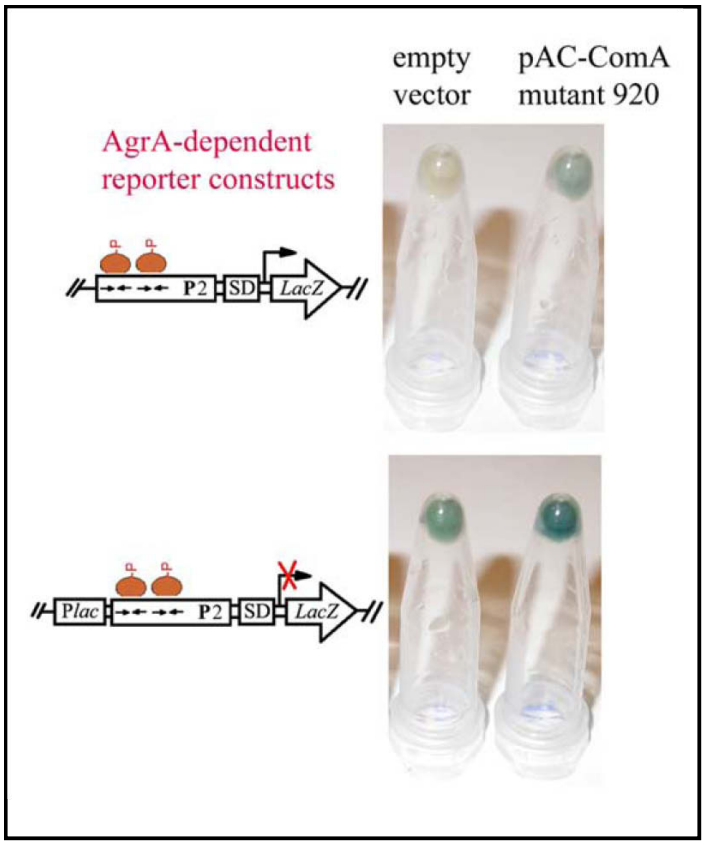

Figure 15: Induction (top) and repression (bottom) of LacZ expression by ComA920 variant.

To test the function ComA binding to the Psrf promoter independent of phosphorylation we created a series of truncation of the proteins receiver domain and also made library of random ComA variants by error-prone PCR. The mutant genes were transformed into *E. coli* strains harboring either pUC-Plac-Psrf-SD-LacZ (ComA binding elements; alternative construct having LacZ under positive control of Psrf was also tested) or pUC--P2-SD-LacZ (AgrA P2 promoter sequence). No repression (or induction in the alternative construct) of LacZ was observed with any of the mutants with the Psrf promoter region. However, surprisingly one variant (ComA920 with A98T, I117K, K140R and N171S) containing several point mutations induced LacZ expression from the *S. aureus* P2 promoter shown in Fig. 15. Conversely, these mutant repressed expressions in the construct were LacZ expression is under negative control. These results have been confirmed by measuring β-galctosidase activities in *E. coli* cells co-expressing ComA920 and the P2-SD-LacZ construct (positive control).

A structural model of ComA was built based on the structure of the nitrate/nitrite response regulator protein NarL from *E. coli* (PDB: 1a04). Three of the mutations in the constitutive ComA variant (at least for the P2 promoter of the Agr system) are located in the receiver domain and two of these mutations are found in an interdomain region known to be

important for shielding the DNA binding domain when the active site Asp in the receiver domain is not phosphorylated [Allen et al. Genetic evidence that the a5 helix of the receiver domain of PhoB is involved in interdomain interactions. (2001) J. Bact. 183:2204-2211.] (Figure 16).

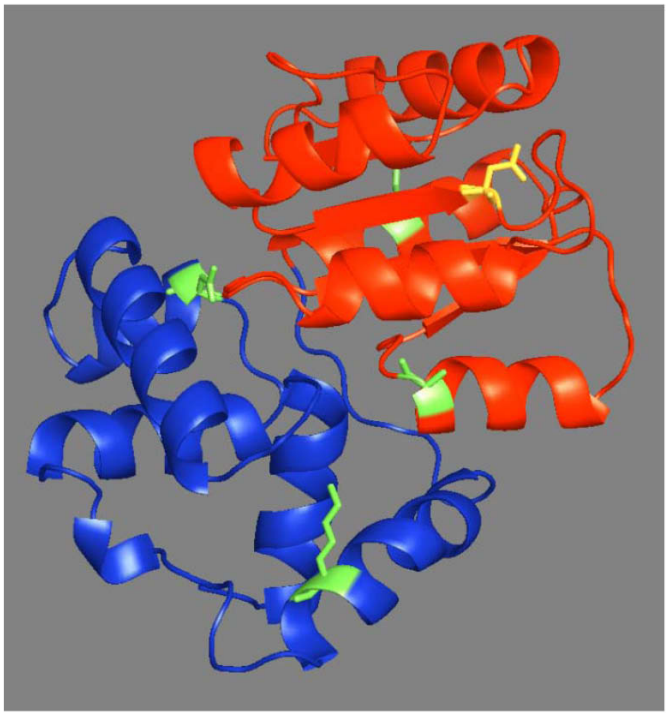

Figure 16: ComA model showing receiver domain (red), DNA binding domain (blue) and Asp phosphorylation site (yellow). Mutations in constitutive variant ComA920 are shown in green.

The experiments with the ComA920 variant suggest that this variant binds to the P2 promoter region of *S. aureus* (Agr system) but not to the *B. subtilis* Psrf promoter (Com system). Gel mobility shift assays were performed to confirm these results with ComA920 and also verify no binding of wt ComA to Psrf. The Psrf and P2 promoter regions were amplified from the reporter constructs and the DNA biotinylated. Shift assays were performed with ComA isolated from AIP exposed and unexposed *E. coli* cells harboring pUC-ComA-ComX. Fig. 17 shows no difference in mobility of the Psrf fragment between the two samples. The same was observed with the P2 promoter fragment (not shown). However, the constitutive ComA920 variant clearly causes a shift in mobility of the P2 fragment without AIP addition to the culture, indicating DNA binding. No mobility shift was observed with ComA and the Psrf promoter fragment. The mobility gel shift experiments therefore confirm the in vivo results in *E. coli* cells expressing the P2 and Psrf reporter constructs and ComA+ComP or ComA920.

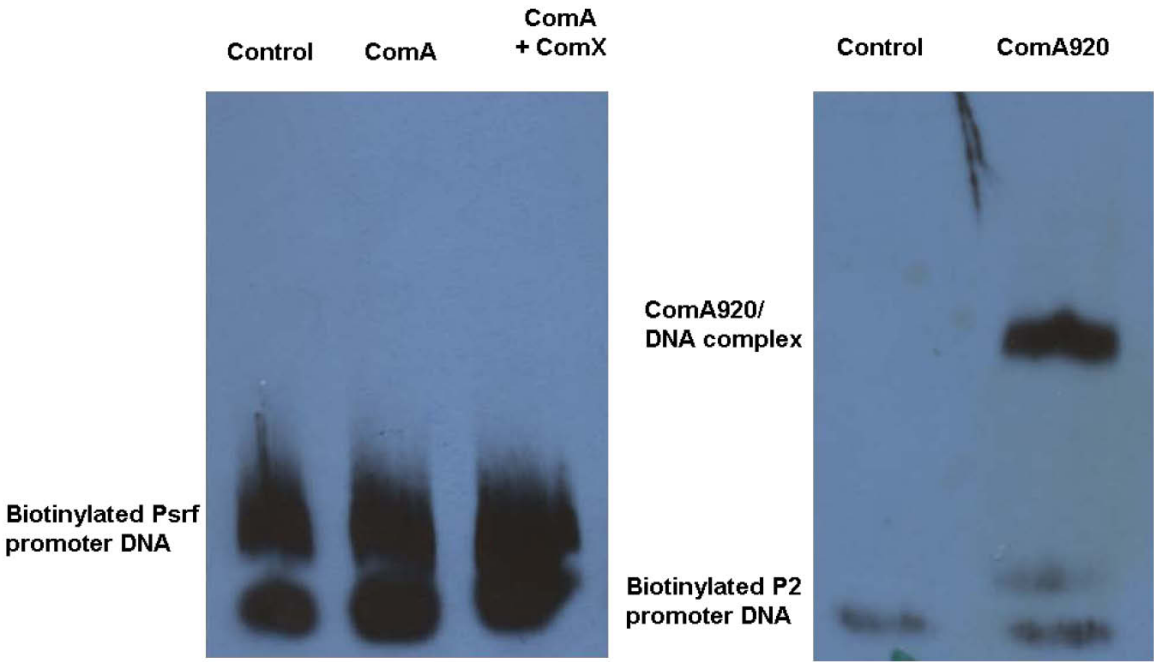

Figure 17: Gel mobility shift assays with ComA and ComA920.

4. Engineering of functional *B. subtilis* sender and receiver cells

Based on the recent findings published by Grossman's group, we have designed a new ComA consensus binding region that contains the third new recognition element (Fig. 18).

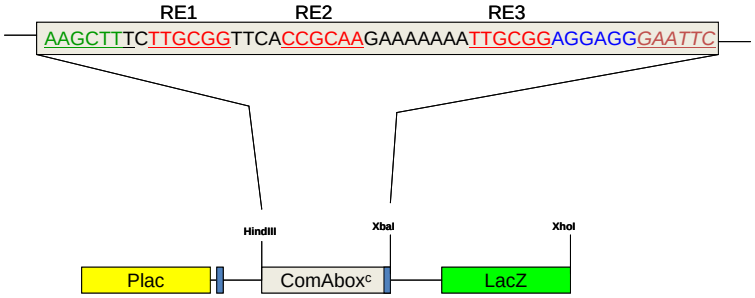

Fig. 18: Redesigned ComA binding box in LacZ reporter construct.

This new construct allowed for the first time the demonstration of a functional gram postive QS circuit in *E. coli*. Fig. 19 shows that the addition of purified ComX (autoinducing peptide) to *E. coli* cultures results in repression of LacZ expression (negative control) as measured by a decrease of β-galactosidase activity monitored in *E. coli* lysates. Repression LacZ expression by ComX occurs in a dose dependent manner. Control cultures without IPTG induction required for expression of the ComA response regulator and ComP sensor kinase do not show this behavior. Also, no ComX dependent repression is observed in cells that do not express ComA or ComP. *E. coli* cells expressing the constitutive mutant ComA920 show as expected repression of LacZ regardless of ComX addition.

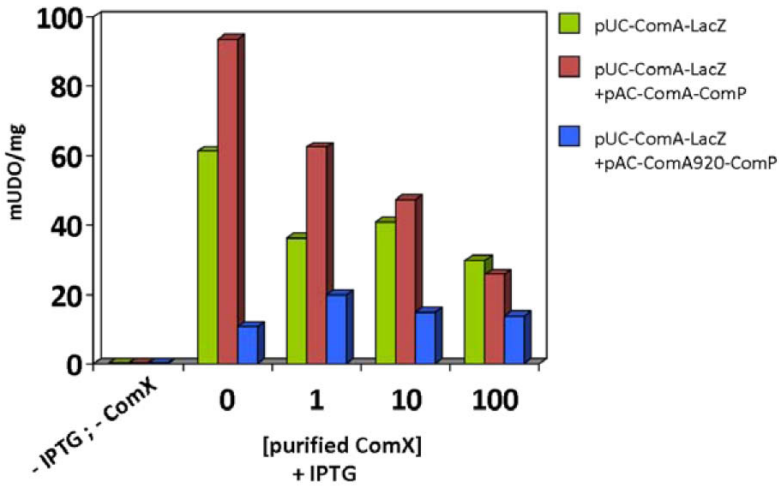

Figure 19: Negative control of LacZ expression by ComX in *E. coli* cells co-expression ComA binding box-LacZ reporter construct as well as ComA and ComP. Measurement of b-galactosidase activity in lysed *E. coli* cells culture grown in the presence of various concentration of purified ComX autoinducing peptide.

To demonstrate the function of the gram positive circuit *in vivo*, purified ComX was spotted on a filter piece next to *E. coli* receiver (harboring the reporter construct, response regulator and sensor histidine kinase) cells grown on agar plates (Fig. 20).

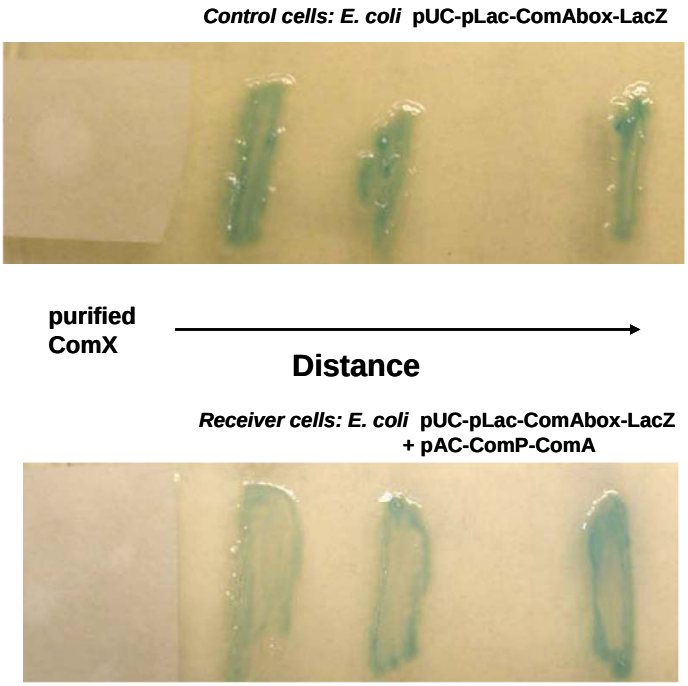

Figure 20: Repression of LacZ expression in receiver cells by ComX diffusing from a filter piece.

Repression of LacZ expression is observed in receiver cells grown closest to the filter piece as the result of ComX diffusion. Control cells lacking response regulator and sensor histidine kinase do not show this behavior. Finally, function of a gram positive sentinel circuit consisting of *E. coli* sender and receiver cells is demonstrated in Fig. 21.

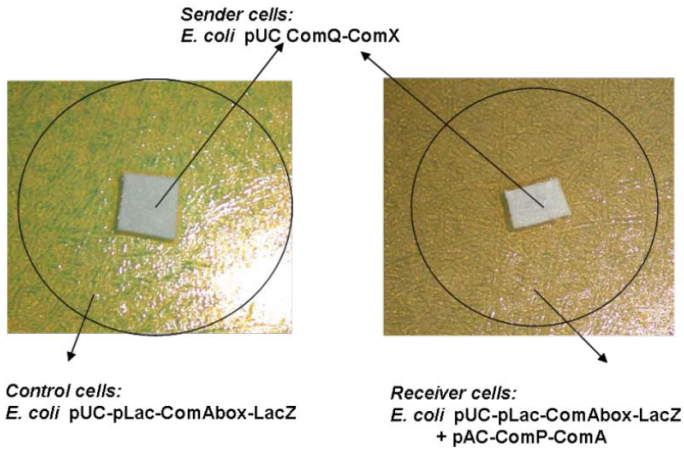

Figure 21: Gram positive sentinel circuit consisting of engineered *E. coli* sender and receiver cells. Secretion of ComX by the sender cells represses LacZ expression by receiver cells compared to control cell

b) Significance

The above discussion signifies that all the constructs are made for both the Agr and Com system. Our results with ComA920 and the gel mobility shift assays indicate that failure of ComA binding to P*srf* promoter represent the bottleneck for the reconstitution of functional receiver cells based on the *Bacillus subtilis* QS system. The P*srf* promoter region amplified for the design of the reporter constructs was based on ComA binding studies reported by Dubnau's group (J. Bacteriol/ 175:3182-3187), which became the foundation for subsequent studies on the *Bacillus* Com QS system. However, very recently Grossman's group published a new study in which they investigated the necessary binding region and sequence for ComA binding (J Mol Biol. 2008 Aug 29;381(2):261-75). Promoter regions of several genes known to be regulated by ComA were compared and a previously unrecognized third consensus binding box was identified and found to be absolutely required for ComA binding. In addition, the authors showed that ComA binding to the Psrt promoter region is rather weak and absolutely requires the presence of all three binding elements plus a duplicated binding region with another set of binding elements. Our Psrf promoter region was based on the original Dubnau publication and did not contain the recently identified third binding element which would explain why we never observed ComA binding in *E. coli* (i.e. LacZ induction or

repression) and *in vitro*. Curiously, inspection of the P2 promoter region led to the identification of regions that show resemblance to the ComA binding regions which may explain why constitutive variant ComA920 binds to the P2 region.

Evolution of highly sensitive and specific detectors

a) Accomplishments

The availability of detectors that function with high specificity and sensitivity is critical to the success of the pathogen sense-and-destroy system. The goal of this work is to evolve the *Pseudomonas aeruginosa* quorum sensing transcription factor LasR to respond to the signal molecule $3OC_{12}HSL$ with higher sensitivity and specificity. A previously described dual-selection system was used to identify three variants with minimal signal-independent activity. Two of these variants, designated 1-6 and 1-9, were studied in more detail. The response to various acyl-homoserine lactone signal molecules was determined and compared with the wild-type LasR.

Table 1 – EC_{50} values for various AHLs. All values shown are nM.

Variant	3OC12-HSL	3OC8-HSL	3OC6-HSL	C8-HSL	C10-HSL	C14-HSL
pLasR	1.5	160	107,000	5400	640	100
pLasR 1-6	1.1	4700	N.D.	N.D.	2800	150
pLasR 1-9	2.4	530	780,000	21,000	1400	110

Table 2 – Specificity values (EC_{50} for given AHL/EC_{50} for 3OC12-HSL).

Variant	3OC8-HSL	3OC6-HSL	C8-HSL	C10-HSL	C14-HSL
pLasR	107	71,000	3600	427	67
pLasR 1-6	4270	N.D.	N.D.	2550	136
pLasR 1-9	221	325,000	8750	580	46

These data show that the two evolved variants have increased specificity compared to the wild-type protein. In particular, variant 1-6 has greatly increased specificity with respect to short-chain AHLs like 3OC8-HSL, 3OC6-HSL, and C8-HSL. It shows a much more modest increase in specificity towards longer chain AHLS such as C10-HSL and C14-HSL.

Variant 1-6 contains two amino acid substitutions, V83A and V221A. Single mutants containing each of these substitutions were constructed and tested for response to AHLs. Interestingly, the two single mutants had opposite effects with respect to short and long-chain AHLs. The V83A mutant had increased specificity toward 3OC8-HSL 30-fold, but similar

specificity to wild-type toward C14-HSL. The V221A mutant had no significant change with respect to specificity toward 3OC8-HSL and a 3-fold increase in specificity toward C14-HSL. Further directed evolution experiments were performed in order to improve specificity toward long-chain AHLs. Error-prone PCR libraries were constructed using pLasR 1-6 as the parent sequence and dual-selection was performed with 1 nM 3OC12-HSL during ON selection and 10 nM C14-HSL during OFF selection. No variants with significantly improved specificity were identified in these experiments. Wild-type error-prone libraries were also subjected to dual-selection and again no improved variants were identified.

b) Significance

LasR variants with greatly improved specificity have been identified. These variants are an important step toward the goal of developing highly sensitive and specific sensors for use in the sense-and-destroy system. Further improvements are needed to reduce LasR response to non-cognate long-chain AHLS, like C14-HSL and C10-HSL. This is presumably a difficult problem due to significant chemical similarity between these AHLs and the cognate signal, 3OC12-HSL. However, though random mutagenesis has not been able to solve this problem to date, the availability of crystal structure data for the ligand-binding domain of LasR may allow targeted mutagenesis to provide solutions to the specificity problem.

Future Plans

1. Implement 3OC12HSL signal amplification for improved detection of *P. aeruginosa*.
2. Implement gram-negative destruction system: Fuse secretion tag to *P. aeruginosa*-specific lysin variants and place under control of 3OC12HSL promoter.
3. Integrate E protein and *P. aeruginosa* specific lysin under control of 3OC12HSL promoter.
4. Results indicate that failure of ComA binding to Psrf promoter is the bottleneck for the reconstitution of functional receiver cells based on the Bacillus QS system.
5. Design of new ComA DNA binding region based on very recent studies by Grossman's group (JMB, July, 2008, in press) that show that a previously unrecognized third consensus binding box is critical and absolutely required for ComA binding.

RE1 RE2 RE3

TC**TTGCGG**CATC**CCGCAA**GAAACT**TTGCGG**

New ComA consensus DNA binding region with third new recognition element RE3

6. Verify ComA binding to new consensus binding region (gel shift and reporter assays)

7. Optimize receiver cells by optimizing promoter design and by directed evolution of ComP and/or ComA.
8. LasR has been successfully evolved to significantly reduce response to short-chain AHLs while maintaining high sensitivity to 3OC12-HSL
9. Targeted mutagenesis will be used to attempt to further increase LasR specificity, particularly with respect to long-chain AHLs. Residues 79, 80, and 125 are potential targets for saturation mutagenesis because they form the end of the hydrophobic pocket that contacts the long acyl chain of 3OC12-HSL in the crystal structure. Additional bulky residues in these positions may disfavor binding by longer AHLs such as C14-HSL.

An important component of the project is the integration of the different specific aims into one sense-and-destroy platform. To facilitate this objective, we have carefully chosen plasmid systems such that the detection and destruction plasmids for gram-negative bacteria will be compatible with the detection and destruction plasmids for gram-positive bacteria. We will integrate test the gram-positive sense-and-destroy circuits with the gram-negative sense-and-destroy circuits. Finally, we will also integrate evolved Las components and phage lysins into the detection and destruction circuits developed.

Renewed Aims

Aim 1: Bacterial two-phase, adaptive response, sense-and-destroy system for gram negative pathogens.

In our previous efforts we engineered bacterial sentinel cells that fluoresce when they detect *P. aeruginosa*. Upon pathogen detection, our engineered sentinels respond by producing a novel toxic chimeric protein, CoPy, which specifically kills the pathogen with no apparent effect on the sentinel cells. For the adaptive two-phase response, we will initially develop an immediate, highly specific first line of defense. To export the killer protein outside sentinel cells, FlgM, a naturally secreted protein from a type III secretion system, will be fused to the N-terminal of CoPy. If the first line of defense fails, the sentinel/killer cells will employ a second line of defense that is delayed, massive, less specific, and suicidal. Using transcriptional cascades, we will fine tune the duration of the delayed response. When the pathogen is initially detected, sentinel/killer cells begin to accumulate high levels of two killer proteins, CDAP and CoPy. If the pathogen persists after the initial attack and a certain time period has elapsed, the sentinel/killer cells commit suicide by rupturing their membrane and release large quantities of CoPy and CDAP that have accumulated in their cytoplasm.

Aim 2: Bacterial detection and destruction of gram-positive pathogens:

During the previous project period we succeeded in demonstrating the first functional gram-positive sentinel circuit (*B. subtilis* Com system) in *E. coli*. In addition to the *B. subtilis* Com receiver/sender cell constructs, we also began to engineer receiver and sender *E. coli* cells for the detection of *Staphylococcus aureus* using components of its Agr QS system. In this project we will implement and optimize three gram-positive QS circuits in *E. coli* for the detection of the pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Bacillus subtilis*, as a model gram-positive QS system. These three QS systems are representative of different known types of peptide mediated QS in gram-positive bacteria and the creation of these sentinel cells we will enable us develop design principles for the engineering of additional sentinels for other gram positive pathogens. Engineering of these gram-positive QS circuits will likely require additional molecular and biochemical studies as our previous efforts on the *B. subtilis* system showed, resulting in improved scientific understanding of these pathways. To create modular sentinel circuits with exchangeable sensor components we will explore the creation of chimeric histidine kinases with swapped trans-membrane receptor and cytoplasmic output domains. With one prototype gram positive QS circuit now available we will proceed with the design of a prototype gram positive pathogen destruction circuit for *B. subtilis*. Circuit design will follow the approach in Aim 1. *B. subtilis* phage lysins will be secreted by *E. coli* killer cells via type II (lysin-FlgM fusion) or type III (lysin-OmcA fusion) for an immediate and specific response. E protein expression will lyse *E. coli* for massive lysin release. All circuit components will be created based on the BioBrick standard so that they can be easily swapped into the amplification and response circuits of Aim 1.

Aim 3: Ultrasensitive, sentinel/killer mammalian cells:

We will genetically engineer mammalian cells to detect *P. aeruginosa* and then kill it by secreting antimicrobial peptide. For this purpose we will design mammalian cells that detect 3OC12HSL produced by PAO-1 in early stages of growth. We will create ultrasensitive sentinels by constructing genetic signal amplifiers capable of detecting trace quantities of the autoinducer produced by the pathogen. Upon pathogen detection, the mammalian cells respond by producing and secreting highly specific bacteriocins developed in Aim 1. These

bacteriocins will be optimized for mammalian expression and adapted for export using mammalian secretory pathways.

Significant Problems

Gram-negative sense and destroy pathway (Princeton University)

Export of lysin into the system from the sentinel cells without killing themselves.

Gram-positive sense and destroy pathway (U of M)

1. Induction of EGFP reporter expression by Receiver cells not yet observed because :

    a. Psrf promoter containing ComA binding boxes not functional in E. coli (likely).

    b. ComP-ComA signaling cascade not yet functional in E. coli (likely – ComP not overexpressed).

    c. ComX not effectively synthesized by Sender cells (unlikely, has been shown to work in E. coli)

Evolution of highly sensitive and specific detectors (Caltech)

1. Dual selection system was not functional when the *lux* box was replaced with a *las* box

    a. LasR activity in the absence of signal is too strong, resulting in resistance to chloramphenicol and sensitivity to carbenicillin when no signal is present

Strains contaning pLasR and plasCAT were resistant to 150 mg/mL Cm in the presence of 0.1 nM 3OC12-HSL (the lowest concentration tested)

Strains contaning pLasR and plasBLIP were inviable on 100 mg/mL Amp regardless of the presence or absence of signal in the media

    b. The regulatory sequences and/or plasmid copy number need to be modified to generate a functional system